ARTICLE IN PRESS

Molecular Ther. 13(4):776-785 (2006)

doi:10.1016/j.ymthe.2005.10.019

ARTICLE

DNA Vaccines Increase Immunogenicity of Idiotypic Tumor Antigen by Targeting Novel Fusion Proteins to Antigen-Presenting Cells

Agnete B. Fredriksen,[1,*] Inger Sandlie,[2] and Bjarne Bogen[1,*]

[1]Institute of Immunology, University of Oslo, Rikshospitalet and Rikshospitalet University Hospital, Sognsvannsvn. 20, 0027 Oslo, Norway
[2]Department of Molecular Biosciences, University of Oslo, P.O. Box 1050 Blindern, 0316 Oslo, Norway

*To whom correspondence and reprint requests should be addressed. Fax: +47 23073510. E-mail: a.b.fredriksen@medisin.uio.no or bjarne.bogen@medisin.uio.no.

Naked DNA vaccines have a number of advantages over conventional vaccines, but induce only weak immune responses. We have here investigated if this inadequacy may be overcome by inducing muscle to secrete fusion proteins with the ability to target antigen-presenting cells (APC). The novel targeted vaccines are homodimers with (i) two identical single-chain fragment variable (scFv) targeting units specific for MHC class II molecules on mouse APC, (ii) a human Ig hinge and C_H3 dimerization unit, and (iii) two identical scFv tumor antigenic units (idiotypes) from B cell cancers. After plasmid injection and electroporation of mouse muscle, secreted vaccine proteins (vaccibodies) delivered idiotypic tumor antigen to APC in draining lymph nodes for induction of T and B cell responses that protected mice against tumor challenges with a multiple myeloma (MOPC315) and a B cell lymphoma (A20). Targeting to APC was essential for these effects. The results show that immunogenicity of plasmid DNA vaccines can be increased by inducing muscle to secrete proteins that target antigen to APC.

Key Words: immunotherapy, naked DNA, vaccine, targeting, antigen-presenting cells electroporation, cancer

Introduction

Naked DNA vaccines have a number of advantages [1,2]; however, a serious drawback is poor immunogenicity of encoded antigens [3–6]. The latter deficiency can be partly overcome by subjecting plasmid-injected muscle to electroporation [7–9] or co-injection of plasmids that encode cytokines or chemokines [10,11] for attraction of dendritic cells [12].

Targeting to APC is particularly efficient for stimulation of $CD4^+$ T cells that recognize peptides presented on MHC class II molecules [13–16]. Activated $CD4^+$ T cells have a crucial role in providing help to B cells and $CD8^+$ T cells, facilitating a broad immune response to antigen. We have here explored if targeting of protein antigen to APC may improve immunogenicity of plasmid DNA vaccines. To this end we have developed novel Ig–antigen fusion molecules.

As antigen in these studies, we have used V regions of monoclonal Ig produced by B cell tumors. V regions express antigenic determinants, called idiotopes (Id), which are highly tumor specific for individual B cell cancers. Immunization with monoclonal Ig, i.e., Id vaccination, induces protection against tumor challenges in mice [17] and has entered clinical trials, with encouraging results [18,19]. Tumor protection may be conferred by Id-specific antibodies [20,21] and Id-specific T cells [22,23].

Because monoclonal Ig V regions are very weak antigens, a number of different approaches and adjuvants, including DNA immunization, have been employed to enhance efficiency of Id vaccination. Thus, injection of Ig genes as naked plasmids induced anti-Id antibody responses and tumor protection [21,24]. Moreover, monoclonal antibodies or scFv have been genetically conjugated to GM-CSF [25], chemokines [26], CD40L [27], tetanus toxin fragment C [28], and IL-1β [29] and used as protein vaccines [25–29] or DNA vaccines [26,28,29] for vaccination against myelomas and B cell lymphomas. However, in these cases, it was unclear whether enhanced uptake of fusion proteins by APC, maturation of APC, or both contributed to enhanced immune responses and tumor protection. In particular, it was not studied whether DNA-injected muscle secreted protein that targeted APC.

1525-0016/$30.00

 doi:10.1016/j.ymthe.2005.10.019

We here describe a novel vaccine reagent and demonstrate that naked DNA vaccination and targeting of protein to APC may be combined to enhance tumor-protective Id-specific immune responses. The experiments were performed both in a B cell lymphoma model (A20) and in a multiple myeloma model (MOPC315). The latter model has the advantage that a well-defined MHC class II-restricted T cell idiotope has been described in the V region of the myeloma protein $\lambda 2^{315}$ Ig L chain (aa 91–101) [30,31]. Moreover, $CD4^+$ T cells specific for this Id ($\lambda 2^{315}$), presented by the I-E^d class II molecule, have been described to confer protection against a tumor challenge with MOPC315 cells [22,23].

RESULTS

Design, Genetic Construction, and Functional Characterization of Targeted Vaccines

The designed molecules are homodimers, each chain consisting of a targeting unit, a dimerization unit, and an antigenic unit (Fig. 1A). As N-terminal targeting units, we used variable regions ($V_H + V_L$ connected by a flexible linker; i.e., scFv) from mAbs specified below. The dimerization unit consisted of a shortened hinge region (h1 and h4 exons) and a C_H3 domain from human γ3 chains. The C-terminal antigenic unit contained scFv from mouse B cell tumors specified below. The two chains were expected to homodimerize by hydrophobic interactions between C_H3 domains and disulfide bonds forming between cysteines in the hinge (Fig. 1A). The construct is shown in Fig. 1B.

In the functional experiments to follow, we employed a scFv from the 14-4-4S mAb specific for MHC class II (I-E) molecules as a targeting unit. As a nontargeted control, we made a construct in which the I-E-specific scFv was exchanged with hapten 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP)-specific scFv from the B1-8 mAb. As antigenic units, we used tumor-specific scFv from the BALB/c MOPC315 myeloma and the A20 B lymphoma cells. Vaccine protein secreted by transfected cells had a size and homodimeric structure as predicted, and the targeting and antigenic units were functional since they bound antigens and antibodies as expected (Fig. 2).

DNA Injection and Electroporation of Muscle Induce Expression of Functional Vaccine Proteins in Serum

After injection of 50 μg plasmid into quadriceps of BALB/c mice, immediately followed by electroporation to enhance expression, we found NIP-specific protein in the range 200–750 ng/ml in serum at day 14. In striking contrast, we did not detect I-E-specific protein (Fig. 3A). The latter finding could be explained if the I-E-specific vaccine were functional *in vivo* and therefore bound I-E^d MHC class II molecules expressed on APC of BALB/c (H-2^d) mice. To test this, we compared serum levels of I-E-specific protein in two pairs of mice with similar non-MHC backgrounds and that either express I-E (BALB/c and B10.D2, both H-2^d) or do not (BALB.B and C57Bl/10, both H-2^b). I-E-specific proteins were readily detected in serum of BALB.B and C57Bl/10 mice in the range 200–600 ng/ml, while they were not detected in BALB/c and B10.D2 mice. Thus, on two different non-MHC backgrounds, we showed that I-E-specific proteins are produced, but bind MHC class II molecules if present (Fig. 3B).

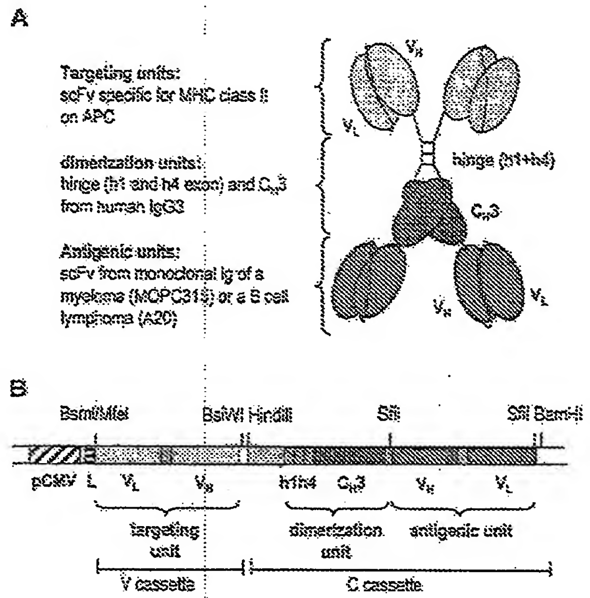

FIG. 1. Design and construction of targeted vaccines. (A) The vaccine proteins are homodimers. Each chain is composed of an amino-terminal scFv ($V_L + V_H$) targeting unit (yellow) that binds MHC class II (I-E^d) molecules on APC, a dimerization unit composed of a shortened human Ig hinge (gray) and C_H3 domain (blue), and an antigenic unit corresponding to tumor-specific scFv from monoclonal Ig of a multiple myeloma (MOPC315) or a B cell lymphoma (red). As a nontargeted control, the MHC class II-specific scFv was replaced with a hapten (NIP)-specific scFv. An MHC class II-restricted $CD4^+$ T cell epitope is located in the $V\lambda 2^{315}$ (aa 91–101) of $scFv^{315}$. (B) Gene construct. The targeting unit (yellow) is inserted into the V cassette of the pLNOH2 vector. The dimerization unit, composed of h1 and h4 hinge exons (gray) and the C_H3 exon of human γ3 (blue), is linked to the antigenic unit (red) and inserted together in the C cassette of pLNOH2. The $(G_4S)_3$ linkers (orange) and GLSGL linker (green) are indicated. The gene is expressed from a CMV promoter (hatched) and a leader sequence (striated) of the pLNOH2 vector (uncolored). Upstream of h1 is an intronic sequence (light gray).

We detected hardly any vaccine proteins in the absence of electroporation (Fig. 3C), consistent with previous reports demonstrating that electroporation enhances expression of plasmid genes [7–9]. A titration experiment indicated that 3.12 μg, divided on two injected quadriceps muscles, was the minimum amount for generation of detectable levels of vaccine protein in sera. Levels of protein increased with the amount of injected plasmids up to the largest dose tested (200 μg) (Fig. 3D). We detected vaccine proteins in maximum amounts in serum at day 7; thereafter the level gradually declined until day 170, when

doi:10.1016/j.ymthe.2005.10.019

nothing could be detected (Fig. 3C). This demonstrates that muscle produces targeted vaccines for prolonged periods of time.

Targeting of Vaccine Protein to APC is Essential to Induce Antibody Responses

Within 14 days, BALB/c (I-E$^+$) mice injected once with I-E-specific plasmid developed an antibody response against V regions of the M315 myeloma protein (Fig. 4A). Antibodies were Id-specific since they did not bind J558 IgA with different V regions (data not shown). Levels of anti-Id antibodies reached a maximum 60 days after vaccination, after which levels slowly declined until they reached the lower detection limit at day 170. Only small amounts of IgM anti-Id^{315} could be detected and only very early in the response. IgG2a dominated for the first 50 days; thereafter, more IgG1 was detected (Fig. 4B). Repeated plasmid injections every third week boosted anti-Id antibody responses (compare Figs. 4A and 4H); moreover, IgG2a remained the dominating isotype within a time span of 63 days (data not shown).

Targeting of protein to APC was crucial for induction of anti-Id antibody responses because injection of NIP-specific plasmids induced only delayed, weak antibody responses (Figs. 4A and 4B). In fact, when we plotted levels of anti-Id Ab vs. vaccine protein in an x–y plot, a very clear relationship came out: mice injected with I-E-specific plasmids had no vaccine protein in serum but potent anti-Id antibody responses, while mice injected with NIP-specific plasmids had detectable levels of vaccine proteins in serum, but no anti-Id antibodies (Fig. 4C). This result suggested that targeting of DNA-encoded antigen to I-E positive APC greatly enhances antibody responses.

To test further the effect of targeting vaccines to I-E MHC class II molecules, we performed an experiment in MHC congenic mice. BALB.B mice (H-2^b), which are similar to BALB/c (H-2^d) except that they lack I-E MHC class II molecules, had no detectable anti-Id^{315} antibodies following DNA injection of I-E-specific plasmids. By contrast, a strong response was observed in BALB/c mice that express I-E (Fig. 4D). Similarly, C57Bl/10 mice (H-2^b) had no detectable anti-Id^{315} response, while B10.D2 mice (H-2^d) did. These results show that targeting of antigen to class II molecules on APC is essential for induction of anti-Id antibodies.

Levels of anti-Id antibodies increased with amounts of injected plasmids. Moreover, higher amounts of

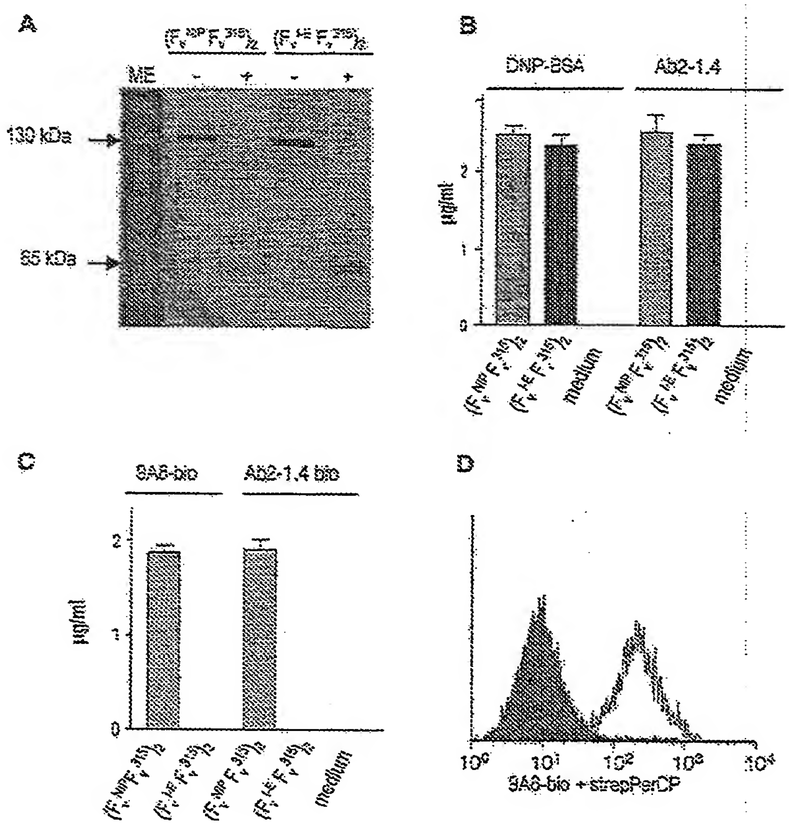

FIG. 2. M315-vaccine proteins are secreted as functional homodimers. Targeted and nontargeted vaccines are denoted $(F_v^{I\text{-}E}\ F_v^{315})_2$ and $(F_v^{NIP}\ F_v^{315})_2$, respectively. (A) 10% SDS–PAGE and autoradiography of metabolically labeled protein immunoprecipitated from culture supernatants of NS0 transfectants. Disulfide bonds were reduced (+) or not reduced (–) with mercaptoethanol (ME). (B) Conformational correctness of antigenic scFv^{315} in the vaccine format was tested by the ability to bind the hapten DNP (for which M315 is specific) and the Ab2-1.4 mAb (specific for idiotype of Fv^{315}) in ELISA. Vaccine proteins were detected with biotinylated Vλ-specific 9A8 mAb, which binds Fv^{315} (Vλ2) and Fv^{NIP} (Vλ1), but not Fv$^{I\text{-}E}$ (Vκ). (C) Maintenance of NIP specificity was measured by binding to NIP–BSA in ELISA, detected by biotinylated 9A8 or Ab2-1.4 mAbs. (D) Maintenance of I-E^d MHC class II specificity in targeted vaccines. Staining of BALB/c (H-2^d) CD19$^+$ splenocytes with class II-specific protein (open histogram) and the nontargeting (NIP)-specific protein (filled histogram). Bound proteins were detected with fluorochrome-labeled 9A8 mAb that binds Fv^{315}.

doi:10.1016/j.ymthe.2005.10.019

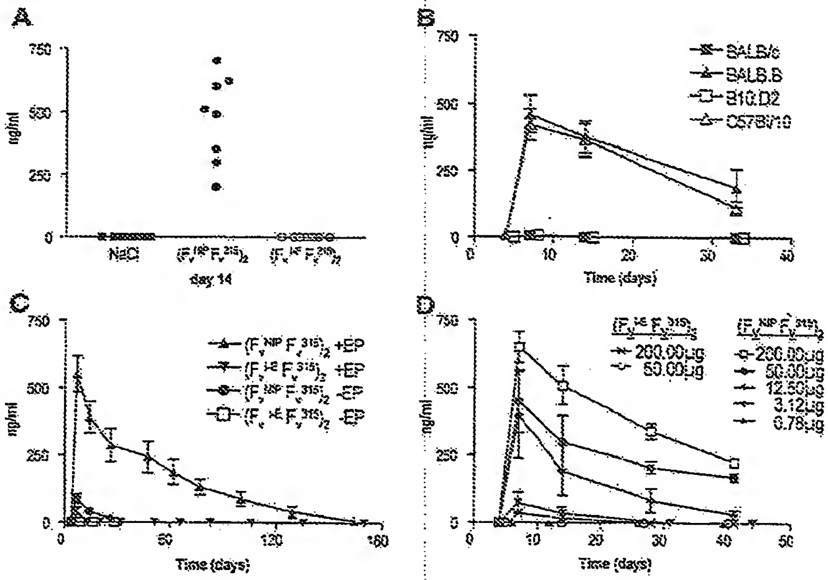

FIG. 3. M315-vaccine protein in serum after intramuscular injection of naked plasmids. Vaccine proteins were measured in a sandwich ELISA that detects both $(F_v^{NIP} F_v^{315})_2$ and $(F_v^{I-E} F_v^{315})_2$ proteins. (A) Vaccine proteins in sera of BALB/c mice, which express I-E MHC class II molecules, tested 14 days after injection of 50 µg plasmids and electroporation. (B) Vaccine protein present in sera after injection of 50 µg of MHC class II-specific plasmids, followed by electroporation, in strains of mice that have similar non MHC genetic background but that differ in the absence or presence of I-E class II molecules [BALB/c (I-E$^+$) vs. BALB.B (lacks I-E); B10.D2 (I-E$^+$) vs. C57Bl/10 (lacks I-E)]. (C) Vaccine protein present in sera after injection of 50 µg plasmids followed (+EP) or not followed (−EP) by electroporation. (D) Serum protein levels after injection of titrated amounts of plasmids and electroporation.

plasmids resulted in earlier detection of antibody responses (Fig. 4E). The lowest dose that induced anti-Id Abs was 3.12 µg of I-E-specific plasmids (Fig. 4E). The antibody responses were dependent upon *in vivo* electroporation, since BALB/c mice that received class II-specific plasmids in the absence of electroporation had hardly any anti-Id antibody response (Fig. 4F).

The sustained IgG antibody response (Figs. 4A–4F) indicated an ongoing immunization by the targeted vaccines, which is consistent with protein being present in sera for up to 170 days after a single plasmid injection (Fig. 3). To test this idea further, we vaccinated BALB/c mice with I-E-specific plasmids and blocked binding to I-E molecules by saturating the mice with 14-4-4S mAb, the donor mAb of the scFv of the I-E-specific vaccine construct. While injection of isotype-matched control mAb had no effect, the 14-4-4S mAb completely blocked development of anti-Id antibodies during the 28-day injection period (Fig. 4G). However, 14 days after the last injection of anti-I-E mAb, the mice started to develop increasing amounts of anti-Id antibodies. These results indicate that I-E-specific vaccine proteins are still produced and secreted more than 28 days after vaccination, suggesting a prolonged vaccine effect.

Targeted Vaccines Enhance Stimulation of CD4$^+$ T Cells *in Vitro* and *in Vivo*

Targeted vaccines were 100–1000 times more efficient at presenting the Vλ2^{315} epitope to CD4$^+$ T cells than nontargeted protein, demonstrating that binding to MHC class II molecules on APC induces superior T cell responses *in vitro* (Fig. 5A). Moreover, 8 days after injection of MHC class II-specific plasmids and electroporation, APC in draining lymph node (LN) were primed and able to activate λ2^{315}-specific CD4$^+$ T cells in an *in vitro* proliferation assay (Fig. 5B). We observed similar results on T cell activation also *in vivo* because λ2^{315}-specific CD4$^+$ T cells in draining LN had an increased expression of the early activation marker CD69 (Fig. 5C) and proliferated (incorporated 5-bromo-2′-deoxy-uridine (BrdU), Fig. 5D). Both priming of APC and T cell activation in LN required targeting to APC, because injection of nontargeted NIP-specific plasmids failed in this respect (Figs. 5B–5D). We found primed APC and activated T cells only in draining lumbar and sacral LN and not in nondraining mesenteric counterparts.

Targeted Vaccines Induce Protective Immunity against the MOPC315 Myeloma and the A20 B Cell Lymphoma

Targeted class II-specific plasmids induced significant protection in vaccinated BALB/c mice against a sc challenge with MOPC315 cells, while nontargeted NIP-specific plasmids did not, even though both contained Fv^{315} (Fig. 6A, $P < 0.01$). The protection conferred by vaccination with class II-specific plasmids correlated with a pronounced reduction of M315 myeloma protein in serum (Fig. 6B). Close to identical findings were obtained in mice vaccinated three times spaced 3 weeks apart (Fig. 6A); thus, the increased serum levels of anti-Id antibodies in response to repeated vaccination (Fig. 4H) did not increase the protection level compared with a single injection. We observed similar tumor protection also in mice vaccinated with single injections of 200 and 12.5 µg of I-E-specific plasmid, while a single injection of 3.12 µg

doi:10.1016/j.ymthe.2005.10.019

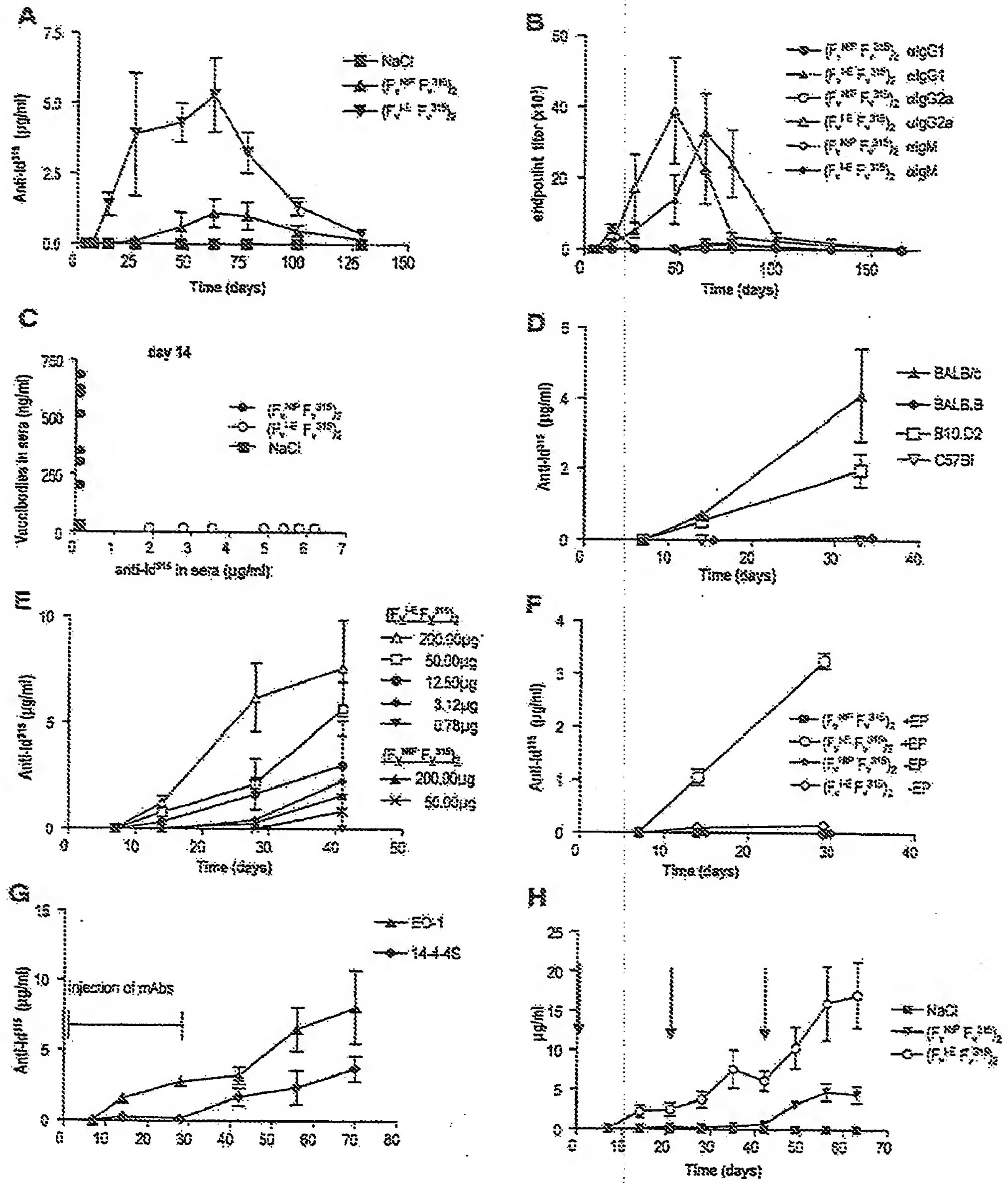

FIG. 4. Production of anti-Fv[315] (anti-Id) antibodies after intramuscular injection of mice with vaccine plasmids. (A) Total anti-Id antibodies (κ^+) binding M315 in ELISA after injection of 50 µg plasmids and electroporation. (B) Titers of IgM, IgG1, and IgG2a anti-Id antibodies in the same sera. (C) Amount of vaccine proteins (y axis) and anti-Id antibodies (x axis) simultaneously detected in sera of individual mice on day 14 after injection of 50 µg nontargeted $(F_v^{NIP}\ F_v^{315})_2$ and targeted $(F_v^{I\text{-}E}\ F_v^{315})_2$ plasmids, and electroporation. (D) Anti-Id antibodies after injection of 50 µg plasmids and electroporation in paired strains of mice that have similar non-MHC genetic backgrounds but that differ in the presence or absence of I-E class II molecules [BALB/c (I-E⁺) vs. BALB.B (lacks I-E); B10.D2 (I-E⁺) vs. C57Bl/10 (lacks I-E)]. (E) Anti-Id antibodies elicited after injection of different amounts of plasmids. (F) Anti-Id antibodies elicited by injection of 50 µg naked plasmids followed (+EP) or not followed (−EP) by electroporation. (G) MHC class II I-E-specific mAb reversibly inhibits induction of anti-Id antibodies by the I-E-specific vaccine reagent. BALB/c mice were injected im with 50 µg MHC class II-specific plasmids $(F_v^{I\text{-}E}\ F_v^{315})_2$ and electroporated. The mice were injected ip three times a week with either 100 µg 14-4-4S (I-E-specific) mAb or 100 µg EO-1 isotype-matched control mAb from day 0 until day 28, as indicated by the bar. (H) Total anti-Id antibodies (κ^+) in mice injected three times with 50 µg plasmids and electroporated on days 0, 21, and 42 (arrows).

doi:10.1016/j.ymthe.2005.10.019

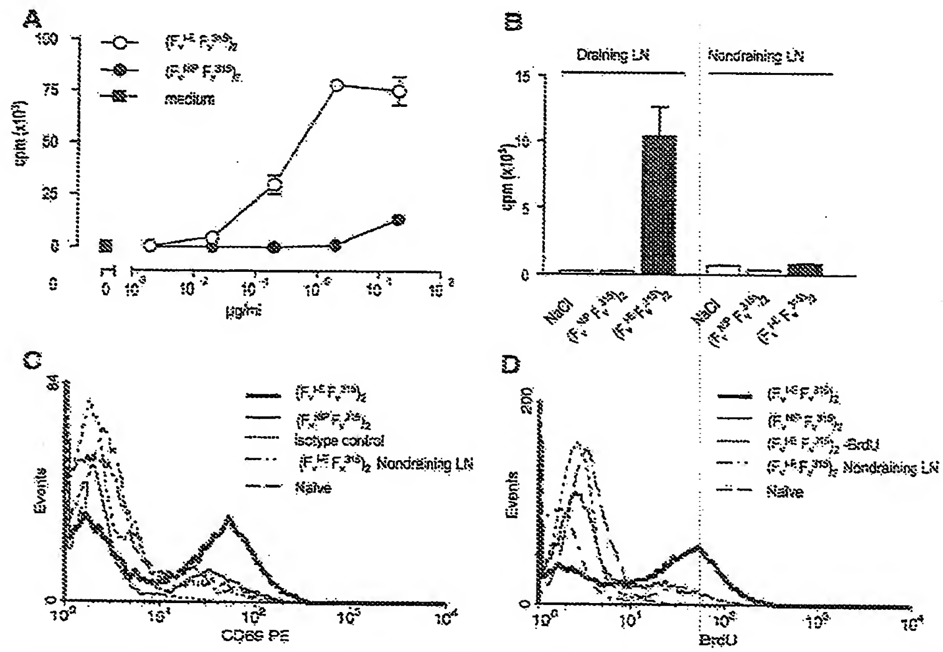

FIG. 5. The vaccine reagent efficiently delivers T cell epitopes *in vitro* and *in vivo*. (A) Irradiated (8 Gy) BALB/c spleen cells were pulsed with titrated amounts of either class II-specific or nontargeting NIP-specific proteins before washing and addition of polarized $V\lambda2^{315}$-specific Th2 cells from TCR-transgenic mice. Proliferation of T cells was measured 3 days later by incorporation of [^{3}H]dTdR. In (B–D), $V\lambda2^{315}$-specific TCR-transgenic SCID mice were injected once with 50 μg plasmids and electroporated. (B) Draining (lumbar and sacral) and nondraining (mesenteric) lymph node cells were isolated 8 days after vaccination, irradiated, and incubated with $\lambda2^{315}$-specific 7A10B2 CD4$^+$ T cells. (C) Draining lymph node cells, taken 10 days after vaccination, were stained for flow-cytometric analysis. Gated TCR clonotype$^+$ CD4$^+$ T cells were analyzed for CD69 expression. Nondraining lymph nodes and naive TCR-transgenic SCID mice served as controls. (D) Vaccinated and naive transgenic mice received BrdU ip and in the drinking water from day 8. On day 14, gated TCR clonotype$^+$ CD4$^+$ T cells in draining and nondraining lymph nodes were analyzed for BrdU incorporation.

plasmid was insufficient to induce protection (data not shown). Electroporation was required since vaccination with 50 μg class II-specific plasmids in the absence of electroporation failed to induce protection (data not shown). The protection induced by targeted vaccines was specific for Fv^{315} because vaccinated mice succumbed to a challenge with another IgA myeloma, J558 (Fig. 6A).

To extend these results to a lymphoma model, with differing antigenic V regions, we genetically replaced the $scFv^{315}$ antigenic unit with $scFv^{A20}$ derived from the BALB/c A20 B cell lymphoma. Mice vaccinated with 50 μg targeted MHC class II-specific A20 plasmids, and electroporated, were protected against a sc challenge with A20 B cell lymphoma cells, while mice vaccinated with the nontargeted A20 plasmids were not (Fig. 6C, $P < 0.01$). Hence, these targeted vaccines appear to be an efficient Id vaccine for both myeloma and B cell lymphoma.

Discussion

We here show that the effect of naked DNA vaccination can be enhanced by the use of plasmids encoding novel Ig-antigen fusion molecules that target APC. Thus, transfection of muscle cells with targeted vaccine genes resulted in tumor antigen-primed APC in draining LN, T and B cell responses, and protection against subsequent tumor challenges with a myeloma and a B cell lymphoma. Electroporation of the muscle DNA injection site, previously shown to enhance gene expression and immune responses [7,8,32], boosted efficiency of the targeted vaccine. Two features may have contributed to efficient targeting to APC: bivalency and lack of binding to Fc receptors (since the designed molecules lack C_H2 domains).

Vaccine protein with hapten (NIP) specificity could be found in serum for as long as 5 months after a single DNA injection and electroporation, with maximum amounts (200–700 ng/ml) being detected as early as 7 days. Thus, muscle cells can synthesize and secrete these vaccine proteins as well as natural Ig [32–34] for prolonged periods of time, both having a complex dimeric structure and interchain disulfide bonds. In previous studies, genes have been delivered with viral vectors [33,34] or plasmids combined with electroporation [32], the latter having the advantage of avoiding immune responses against viral proteins. The prolonged secretion of the vaccine protein

doi:10.1016/j.ymthe.2005.10.019

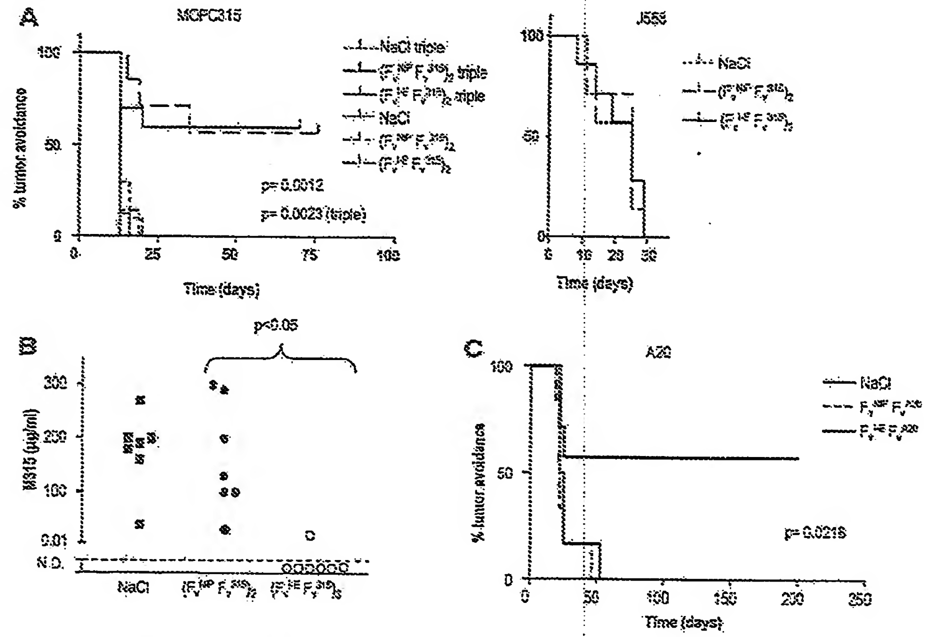

FIG. 6. DNA vaccination with targeted vaccine constructs induces protection to tumor challenges. (A) BALB/c mice were injected and electroporated with 50 µg naked plasmids encoding MHC class II-specific vaccine $(F_v^{I\text{-}E}\ F_v^{315})_2$, nontargeted NIP-specific vaccine $(F_v^{NIP}\ F_v^{315})_2$, or 0.9% NaCl. Fourteen days later, immunized mice were challenged sc with 1.6×10^5 MOPC315 or J558 myeloma as a negative control. In another experiment, mice were vaccinated repeatedly on days 0, 21, and 42 (see Fig. 4H) and challenged with MOPC315 myeloma cells on day 63. A tumor of >3 mm was scored as tumor take. (B) Levels of M315 myeloma protein in the sera of single vaccinated mice on day 18 after MOPC315 challenge. N.D., not detected. (C) BALB/c mice were injected with 50 µg naked plasmids encoding MHC class II-specific vaccine with scFv antigenic unit from the A20 B cell lymphoma $(F_v^{I\text{-}E}\ F_v^{A20})_2$, or nontargeted NIP-specific A20 vaccine $(F_v^{NIP}\ F_v^{A20})_2$, or 0.9% NaCl. Fourteen days later, vaccinated mice were challenged sc with 1.2×10^5 A20 B cell lymphoma cells.

described herein apparently resulted in immunization of long duration extending for at least 28 days and IgG antibodies characteristic of a secondary B cell response. Consistent with this, repeated injections of targeted vaccine plasmids did not alter the level of tumor protection, although the maximum levels of anti-Id antibodies were increased fourfold. If prolonged immunization is unwanted, vaccine production might be abrogated by including an inducible promoter in the construct.

It was essential that injected plasmids encoded protein with the ability to target tumor antigen to APC. This result is consistent with previous findings that targeting of protein to APC is important for induction of immune responses [13,16,35]. The targeting effect is most likely due to enhanced uptake of protein by APC and channeling of antigen into the conventional pathway for MHC class II presentation to $CD4^+$ T cells [35]. Importantly, we found primed APC in draining LN that activated $CD4^+$ T cells. Targeted protein could have loaded local APC in the electroporated muscle, where MHC class II^+ cells of an undefined phenotype have been described [36]. Alternatively, proteins could drain to LN where they might prime resident APC. Plasmids might also have transfected APC in the muscle [37,38] or in the draining lymph node [39,40]; however, this is considered unlikely because in that case nontargeted proteins should also be efficiently presented, which they were not. Since primed APC were detected in TCR-transgenic SCID mice that lack B cells, APC were most likely macrophages or dendritic cells.

The vaccine reagent has been constructed so that the antigenic units should express conformation-dependent antigenic determinants recognized by B cell receptors (BCR) of B cells. We envisage that B cells with a specific BCR will bind the antigenic unit of vaccine proteins, followed by processing and presentation of antigen to helper $CD4^+$ T cells that first have been stimulated by primed dendritic cells or macrophages (see preceding paragraph). B cells could recognize either soluble vaccine protein or protein bound to APC as in B cell–APC synapses [41].

Although targeted protein elicited production of anti-Id antibodies, these might be of little significance in protection against the MOPC315 myeloma cells because antibodies should be blocked by ubiquitous M315 myeloma protein. It might be more important that the targeted proteins potently stimulated Fv^{315}-specific $CD4^+$ T cells since such Id-specific $CD4^+$ T cells have been shown to be tumor protective in this model [22,23,42]. In the A20 B cell lymphoma model, however, anti-Id antibodies might be of greater importance since

doi:10.1016/j.ymthe.2005.10.019

(i) B lymphomas express BCR in their cell membrane, (ii) they secrete little Ig, and (iii) anti-idiotypic antibodies have been shown to have an anti-lymphoma effect [20,21].

The design of the vaccine construct as an assembly of individual cassettes makes it easy to generate novel targeted vaccine molecules. Thus, the targeting unit can be exchanged with scFv of other specificities as well as non-Fv ligands like chemokines (A.B.F. and B.B., unpublished data). The antigenic unit can within weeks be exchanged with another tumor-specific scFv, as shown herein. Moreover, we have produced targeted constructs with scFv from multiple myeloma cells of patients, indicating that individualized, tailor-made vaccines for specific patients may be made in this format (Marianne Frøyland, A.B.F., and B.B., unpublished data). We believe that these molecules combine several factors important for induction of a strong immune response such as bivalency, flexibility, no FcγR binding, and inclusion of both B and T cell epitopes. It may therefore be feasible to construct such molecules for a variety of infectious and cancer diseases, and we denote them as vaccibodies. Based on the present results, immunization with vaccibodies might be considered for immunotherapy of multiple myeloma and B cell lymphoma patients.

Materials and Methods

Mice and Cell Lines

BALB/c mice and $\lambda 2^{315}$-specific MHC class II (I-E^d)-restricted TCR-transgenic mice on a SCID background [23] were bred by Taconic (Ry, Denmark). B10.D2, BALB.B, and C57Bl/10 mice were from Harlan UK Ltd. (Bicester, England). The study was approved by the National Committee for Animal Experiments (Oslo, Norway). The MOPC315.4 (IgA, λ2) [17] and J558 (IgA, λ1) cell lines were from ATCC, while the BALB/c B cell lymphoma A20 (IgG2a, κ) cell line [43] was kindly provided by Dr. Soren Buus (Copenhagen). The 91-101 $\lambda 2^{315}$-specific, I-E^d-restricted $CD4^+$ T cell clone 7A10B2 has been described previously [44].

Construction of Vaccine Molecules

The dimerization unit. The dimerization unit was cloned from a pUC19 vector that contains the h4 hinge exon genetically combined with the C_H3 domain of the hIgG3 subclass [45]. The primers included restriction enzyme sites (underlined): 5′h4, TAGCAAGCTTGGCCAGCGCAGGGAG; 3′C_H3, CAGGCCACCGAGGCCTTTACCCGGAGACAGGGA. The h1 exon was introduced directly upstream of the h4 exon by QuickChange PCR using the following primers (h1 sequence is shown in bold): h1,1, 5′-CTCCCAATCTTCTCTCTGCA**GAGCTCAAAACCCCACTTGGTGACAACTCACACAGAGCCCAAATCTTGTGACAC**-3′, and h1,2, 5′-**GTGTCACAAGATTTGGGCTCTGTGTGAGTTGTGTCACCAAGTGGGGTTTTGAGCTC**TGCAGAGAGAAGATTGGGAG-3′.

The antigenic unit. The antigenic M315 and A20 V region genes were cloned from MOPC315.4 myeloma cells or A20 B cell lymphoma cells, respectively. mRNA was extracted from the cell lines with oligo(dT)-coated magnetic Dynabeads (Dynal AS, Oslo, Norway). First-strand cDNA was synthesized and used as template for PCR amplification of V region genes using specific primers annealing to the exact ends of the V region sequences. The primers included restriction enzyme sites (underlined), linkers (bold), and stop codons (italic). The primer sequences were 5′M315 V_H, GGCCTCAGCGGCCTGGACGTGCAGCTGCAGGAGTCT; 3′M315 V_H, GCCAGAGCCACCTCCGCCAGATCCGCCTCCACCTGAGGAGACTGTGACAGTGGT; 5′M315 V_L, GGCGGAGGTGGCTCTGGCGGTGGCGGATCGCAGGCTGTTGTGACTCAGGAA; 3′M315 V_L, GACGTCGACTAGGACAGTGACCTTGGTTCC; 5′A20 V_H, GGCCTCAGCGGCCTGATGGTCCAACTGCAGCAGTCA; 3′A20 V_H, TCCCGATCCGCCACCGCCAGAGCCACCTCCACCTGAGGAGACTGTGAGTTCGGT; 5′A20 V_L, GGCGGTGGCGGATCGGGAGGAGGCGGTTCTGATGTTGTGATGACGCAGACT; 3′A20 V_L, CAGGCCCTGCAGGCC*TCA*TTTGACTTCCAGCTTTGTGCC.

Antigenic unit + dimerization unit. The V_H and V_L genes were joined by PCR SOEing into a scFv format. The M315 scFv product was then joined to the hinge-C_H3 genes (see above) by PCR SOEing. The product of this reaction was digested with *Hin*dIII and *Sal*I and subcloned into a pUC19 vector. Two *Bam*HI restriction enzyme sites inside the V_H and V_L of M315 were removed by QuickChange PCR using primers BamHI V_L1, ATGCCAACTGGATACAAGAAAAACC; BamHI V_L2, GGTTTTTCTTGTATCCAGTTGGCAT; BamHI V_H1, TGGAACTGGATACGGCAGTTTCC; and BamHI V_H2, GGAAACTGCCGTATCCAGTTCCA. Next, to introduce a stop codon (italic) and a *Sfi*I and a *Bam*HI restriction enzyme site (underlined) downstream of the coding region, a QuickChange PCR was performed using primers 3′V_L stop1, GTCACTGTCCTA*TGA*GGCCTGCAGGGCCGGATCCGTCGACTCTAG, and 3′V_L stop2, CTAGAGTCGACGGATCCGGCCCTGCAGGCC*TCA*TAGGACAGTGAC. This construct was digested with *Hin*dIII and *Bam*HI and subcloned into the C cassette of the expression vector pLNOH2 [46] (Fig. 1B). To exchange the M315 scFv in the antigenic unit with the A20 scFv PCR product, the pLNOH2 plasmid as well as the A20 scFv PCR product was digested with the restriction enzyme *Sfi*I and the A20 scFv was ligated into the digested plasmid containing the targeting and dimerization unit.

The targeting unit. The V region genes that provide specificity for MHC class II were cloned [35] from the 14-4-4S hybridoma, which produces a mAb specific for the Eα chain (determinant Iam.7) of the I-E MHC class II molecule. Specific primers that anneal to the exact ends of the V region sequences, and with tags designed to include restriction enzyme sites (underlined) or linker sequences (bold), were 5′V_L, GACATTCAATTGACACAGTCTTCTCCTGCTTCC; 3′V_L, GCCAGAGCCACCTCCGCCAGATCCGCCTCCACCTTTGATTTCCAGCTTGGTGCC; 5′V_H, GGCGGAGGTGGCTCTGGCGGTGGCGGATCGCAGGTCCAGCTGCAGCAGT; 3′V_H, GACGTACGACTCACCTGAGGAGACGGTGACTGAGG. The rearranged VDJ gene from the B1-8 B cell hybridoma specific for the hapten NIP [47] was combined with a Vλ1 (Celltech) region gene to yield a scFv with NIP specificity. The primers were designed with similar tag sequences as for 14-4-4S, except for the 5′V_L primer. 5′V_L, GGTGTGCATTCCCAGGCTGTTGTGACTCAGGAA; 3′V_L, GCCAGAGCCACCTCCGCCAGATCCGCCTCCACCTAGGACAGTCAGTTTGGTACCT; 5′V_H, GGCGGAGGTGGCTCTGGCGGTGGCGGATCGCAGGTCCAACTGCAGCAGCC; 3′V_H, GACGTACGACTCACCTGAGGAGACTGTGAGAGTGGT. The V_L and V_H were then joined by PCR SOEing, digested with *Mfe*I and *Bsi*WI or *Bsm*I and *Bsi*WI, respectively, and subcloned into the V cassette in the pLNOH2 vector already containing the hinge-Cγ3-$scFv^{315}$/$scFv^{A20}$ genes in the C cassette (Fig. 1B).

Production and Purification of Vaccine Proteins

The pLNOH2 vector that carries complete vaccine reagent genes expressed from a CMV promoter (Fig. 1B) was stably transfected into NS0 cells by electroporation. Vaccine proteins were affinity purified from supernatant on DNP (bound by M315) [48] or NIP–lysine–Sepharose columns (Sigma).

Metabolic Labeling and Immunoprecipitation

NS0 cells transfected with M315-vaccine constructs were labeled for 6 h at 37°C in 1 ml RPMI lacking methionine and cysteine, with [^{35}S]methionine and [^{35}S]cysteine (Amersham). The supernatant was incubated with anti-mouse Vλ (9A8) and precipitated with magnetic beads coated with sheep anti-rat IgG Dynabeads (Dynal AS). Eluted proteins in sample buffer with or without mercaptoethanol were run on a 10% SDS–PAGE. The gels

were fixed, incubated with Amplify (Amersham), dried, and exposed to BIOMAX-MR film (Eastman Kodak Co., UT, USA).

Measurement of M315-Vaccine Protein, Anti-Id Antibodies, and M315 Myeloma Protein by Sandwich ELISAs

Vaccine protein. DNP-BSA, Ab2-1.4 mouse mAb specific for $scFv^{315}$ (i.e., Id^{315}), or NIP-BSA was used as coat, while biotinylated rat anti-mouse $V\lambda$ (9A8 mAb that detects Fv^{315} having a $V\lambda 2$ domain) or biotinylated HP6017 [anti-human IgG (C_H3)] was used as detection mAb. Purified vaccine proteins served as standards.

Anti-idiotypic antibodies with specificity for Fv^{315}. Myeloma protein M315 (IgA, $\lambda 2$) was used as coat and anti-Id Abs were detected by a biotinylated anti-mouse κ mAb (187.1 bio) or biotinylated anti-mouse IgG1, IgG2a, and IgM mAbs (Pharmingen). Ab2-1.4 (IgG1, κ) anti-Id^{315} mAb was used as standard in some of the ELISAs.

M315 myeloma protein. M315 myeloma protein was measured as described [22].

Flow Cytometry

BALB/c splenocytes were incubated with anti-CD19 PE and NIP- or MHC class II-specific vaccine protein. After being washed, bound proteins were revealed by biotinylated 9A8 (anti-$V\lambda$) and streptavidin PerCP. Twenty thousand cells were run on FACSCalibur (BD Biosciences, Mountain View, CA, USA) and analyzed using the WinMDI software.

DNA Vaccination and Electroporation

Six- to 10-week-old BALB/c, BALB.B, B10.D2, and C57Bl/10 mice were anesthetized, injected with plasmids (purified with Endofree Qiagen kit) in the quadriceps muscle, and electroporated, as previously described [32]. Groups consisted of three to seven mice; means ± SEM are given.

Tumor Challenge

MOPC315.4 cells (1.6×10^5) or A20 cells (1.2×10^5) were injected sc in the right flank. Tumor growth was monitored by palpation and by use of a caliper. The mice were sacrificed when the tumor diameter reached 15 mm. Survival curves and statistical analyses were calculated by use of GraphPad Prism 3.0 software (San Diego, CA, USA).

T Cell and APC Assays

In vitro T cell proliferation assay. T cell proliferation assays were performed as previously described [35]. Briefly, irradiated (20 Gy) BALB/c spleen APC (5×10^5 cells/well) were incubated with titrated amounts of vaccine protein for 4 h at 37°C in 96-well flat-bottom microtiter plates. The cultures were then washed three times before addition of polarized $\lambda 2^{315}$-specific Th2 cells (2×10^4/well) derived from TCR transgenic SCID mice [35]. A 91-107 $\lambda 2^{315}$ synthetic peptide was used as a positive control. After 48 h, the cultures were pulsed for 19 h with 1 μCi [^{3}H]dTdR and harvested, and incorporated [^{3}H]dTdR was measured.

In vivo detection of primed LN APC. $\lambda 2^{315}$-specific TCR-transgenic SCID mice [23] were injected im with 50 μg plasmids and electroporated. Eight days later, draining (lumbar and sacral) and nondraining (mesenteric) lymph node cells were treated with collagenase and DNase and irradiated (20 Gy) and 5×10^5 cells/well were incubated with a $\lambda 2^{315}$-specific T cell clone, 7A10B2 [44]. After 48 h, the cultures were pulsed with 1 μCi [^{3}H]dTdR. Incorporated [^{3}H]dTdR was measured after 22 h.

CD69 expression. Ten days after vaccination, draining and nondraining lymph node cells were triple stained with biotinylated GB113 mAb clonotype-specific for the transgenic TCR, APC-conjugated anti-CD4, and PE-conjugated anti-CD69 (Pharmingen). The biotinylated GB113 mAb was detected by streptavidin CyChrome.

BrdU incorporation. Vaccinated mice were given 1 mg BrdU ip on days 8 and 11 and 1 mg/ml BrdU in drinking water starting on day 8. GB113 $CD4^+$ T cells in the draining and nondraining lymph nodes were stained at day 14 with an APC BrdU flow kit (Pharmingen). The cells were run on a FACSCalibur cytometer.

ACKNOWLEDGMENTS

This study has received funding from The Norwegian Cancer Society, The Norwegian Research Council of Norway, and the Multiple Myeloma Research Foundation. We thank Inovio, Oslo, for providing the electroporator. The technical help of Peter O. Hofgaard, Hilde Omholt, and Mona M. Lindeberg is gratefully acknowledged. We are also grateful to Karoline W. Schjetne for many helpful discussions. Keith Thompson critically read the manuscript.

RECEIVED FOR PUBLICATION JULY 22, 2005; REVISED OCTOBER 24, 2005; ACCEPTED OCTOBER 26, 2005.

REFERENCES

1. Donnelly, J. J., Ulmer, J. B., Shiver, J. W., and Liu, M. A. (1997). DNA vaccines. *Annu. Rev. Immunol.* 15: 617–648.
2. Henke, A. (2002). DNA immunization—a new chance in vaccine research? *Med. Microbiol. Immunol. (Berlin)* 191: 187–190.
3. Calarota, S., *et al.* (1998). Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. *Lancet* 351: 1320–1325.
4. MacGregor, R. R., *et al.* (1998). First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J. Infect. Dis.* 178: 92–100.
5. Wang, R., *et al.* (1998). Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 282: 476–480.
6. Timmerman, J. M., *et al.* (2002). Immunogenicity of a plasmid DNA vaccine encoding chimeric idiotype in patients with B-cell lymphoma. *Cancer Res.* 62: 5845–5852.
7. Aihara, H., and Miyazaki, J. (1998). Gene transfer into muscle by electroporation in vivo. *Nat. Biotechnol.* 16: 867–870.
8. Mathiesen, I. (1999). Electropermeabilization of skeletal muscle enhances gene transfer in vivo. *Gene Ther.* 6: 508–514.
9. Mir, L. M., *et al.* (1999). High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc. Natl. Acad. Sci. USA* 96: 4262–4267.
10. Xiang, Z., and Ertl, H. C. (1995). Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity* 2: 129–135.
11. Weiss, W. R., *et al.* (1998). A plasmid encoding murine granulocyte–macrophage colony-stimulating factor increases protection conferred by a malaria DNA vaccine. *J. Immunol.* 161: 2325–2332.
12. Sumida, S. M., *et al.* (2004). Recruitment and expansion of dendritic cells in vivo potentiate the immunogenicity of plasmid DNA vaccines. *J. Clin. Invest.* 114: 1334–1342.
13. Kawamura, H., and Berzofsky, J. A. (1986). Enhancement of antigenic potency in vitro and immunogenicity in vivo by coupling the antigen to anti-immunoglobulin. *J. Immunol.* 136: 58–65.
14. Baier, G., Baier-Bitterlich, G., Looney, D. J., and Altman, A. (1995). Immunogenic targeting of recombinant peptide vaccines to human antigen-presenting cells by chimeric anti-HLA-DR and anti-surface immunoglobulin D antibody Fab fragments in vitro. *J. Virol.* 69: 2357–2365.
15. Snider, D. P., and Segal, D. M. (1987). Targeted antigen presentation using crosslinked antibody heteroaggregates. *J. Immunol.* 139: 1609–1616.
16. Lunde, E., Munthe, L. A., Vabo, A., Sandlie, I., and Bogen, B. (1999). Antibodies engineered with IgD specificity efficiently deliver integrated T-cell epitopes for antigen presentation by B cells. *Nat. Biotechnol.* 17: 670–675.
17. Lynch, R. G., Graff, R. J., Sirisinha, S., Simms, E. S., and Eisen, H. N. (1972). Myeloma proteins as tumor-specific transplantation antigens. *Proc. Natl. Acad. Sci. USA* 69: 1540–1544.
18. Bendandi, M., *et al.* (1999). Complete molecular remissions induced by patient-specific vaccination plus granulocyte–monocyte colony-stimulating factor against lymphoma. *Nat. Med.* 5: 1171–1177.
19. Timmerman, J. M. (2002). Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. *Blood* 99: 1517–1526.
20. Syrengelas, A. D., and Levy, R. (1999). DNA vaccination against the idiotype of a murine B cell lymphoma: mechanism of tumor protection. *J. Immunol.* 162: 4790–4795.
21. Stevenson, F. K., *et al.* (1995). Idiotypic DNA vaccines against B-cell lymphoma. *Immunol. Rev.* 145: 211–228.
22. Lauritzsen, G. F., Weiss, S., Dembic, Z., and Bogen, B. (1994). Naive idiotype-specific $CD4^+$ T cells and immunosurveillance of B-cell tumors. *Proc. Natl. Acad. Sci. USA* 91: 5700–5704.
23. Bogen, B., *et al.* (1995). Naive $CD4^+$ T cells confer idiotype-specific tumor resistance in the absence of antibodies. *Eur. J. Immunol.* 25: 3079–3086.
24. Syrengelas, A. D., Chen, T. T., and Levy, R. (1996). DNA immunization induces protective immunity against B-cell lymphoma. *Nat. Med.* 2: 1038–1041.
25. Tao, M. H., and Levy, R. (1993). Idiotype/granulocyte–macrophage colony-stimulating factor fusion protein as a vaccine for B-cell lymphoma. *Nature* 362: 755–758.

doi:10.1016/j.ymthe.2005.10.019

26. Biragyn, A., Tani, K., Grimm, M. C., Weeks, S., and Kwak, L. W. (1999). Genetic fusion of chemokines to a self tumor antigen induces protective, T-cell dependent antitumor immunity. *Nat. Biotechnol.* 17: 253–258.
27. Huang, H. I., *et al.* (2004). Improved immunogenicity of a self tumor antigen by covalent linkage to CD40 ligand. *Int. J. Cancer* 108: 696–703.
28. King, C. A., *et al.* (1998). DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. *Nat. Med.* 4: 1281–1286.
29. Hakim, I., Levy, S., and Levy, R. (1996). A nine-amino acid peptide from IL-1beta augments antitumor immune responses induced by protein and DNA vaccines. *J. Immunol.* 157: 5503–5511.
30. Bogen, B., Snodgrass, R., Briand, J. P., and Hannestad, K. (1986). Synthetic peptides and beta-chain gene rearrangements reveal a diversified T cell repertoire for a lambda light chain third hypervariable region. *Eur. J. Immunol.* 16: 1379–1384.
31. Bogen, B., and Lambris, J. D. (1989). Minimum length of an idiotypic peptide and a model for its binding to a major histocompatibility complex class II molecule. *EMBO J.* 8: 1947–1952.
32. Tjelle, T. E., *et al.* (2004). Monoclonal antibodies produced by muscle after plasmid injection and electroporation. *Mol. Ther.* 9: 328–336.
33. Noel, D., *et al.* (2002). High in vivo production of a model monoclonal antibody on adenoviral gene transfer. *Hum. Gene Ther.* 13: 1483–1493.
34. Lewis, A. D., Chen, R., Montefiori, D. C., Johnson, P. R., and Clark, K. R. (2002). Generation of neutralizing activity against human immunodeficiency virus type 1 in serum by antibody gene transfer. *J. Virol.* 76: 8769–8775.
35. Lunde, E., Western, K. H., Rasmussen, I. B., Sandlie, I., and Bogen, B. (2002). Efficient delivery of T cell epitopes to APC by use of MHC class II-specific Troybodies. *J. Immunol.* 168: 2154–2162.
36. Gronevik, E., *et al.* (2003). DNA transfection of mononuclear cells in muscle tissue. *J. Gene Med.* 5: 909–917.
37. Weeratna, R. D., Wu, T., Efler, S. M., Zhang, L., and Davis, H. L. (2001). Designing gene therapy vectors: avoiding immune responses by using tissue-specific promoters. *Gene Ther.* 8: 1872–1878.
38. Fu, T. M., *et al.* (1997). Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol. Med.* 3: 362–371.
39. Chattergoon, M. A., Robinson, T. M., Boyer, J. D., and Weiner, D. B. (1998). Specific immune induction following DNA-based immunization through in vivo transfection and activation of macrophages/antigen-presenting cells. *J. Immunol.* 160: 5707–5718.
40. Condon, C., Watkins, S. C., Celluzzi, C. M., Thompson, K., and Falo, L. D., Jr. (1996). DNA-based immunization by in vivo transfection of dendritic cells. *Nat. Med.* 2: 1122–1128.
41. Batista, F. D., Iber, D., and Neuberger, M. S. (2001). B cells acquire antigen from target cells after synapse formation. *Nature* 411: 489–494.
42. Dembic, Z., Schenck, K., and Bogen, B. (2000). Dendritic cells purified from myeloma are primed with tumor-specific antigen (idiotype) and activate CD4+ T cells. *Proc. Natl. Acad. Sci. USA* 97: 2697–2702.
43. Kim, K. J., Kanellopoulos-Langevin, C., Merwin, R. M., Sachs, D. H., and Asofsky, R. (1979). Establishment and characterization of BALB/c lymphoma lines with B cell properties. *J. Immunol.* 122: 549–554.
44. Bogen, B., Malissen, B., and Haas, W. (1986). Idiotope-specific T cell clones that recognize syngeneic immunoglobulin fragments in the context of class II molecules. *Eur. J. Immunol.* 16: 1373–1378.
45. Olafsen, T., Rasmussen, I. B., Norderhaug, L., Bruland, O. S., and Sandlie, I. (1998). IgM secretory tailpiece drives multimerisation of bivalent scFv fragments in eukaryotic cells. *Immunotechnology* 4: 141–153.
46. Norderhaug, L., Olafsen, T., Michaelsen, T. E., and Sandlie, I. (1997). Versatile vectors for transient and stable expression of recombinant antibody molecules in mammalian cells. *J. Immunol. Methods* 204: 77–87.
47. Neuberger, M. S. (1983). Expression and regulation of immunoglobulin heavy chain gene transfected into lymphoid cells. *EMBO J.* 2: 1373–1378.
48. Eisen, H. N., Simms, E. S., and Potter, M. (1968). Mouse myeloma proteins with antihapten antibody activity: the protein produced by plasma cell tumor MOPC-315. *Biochemistry* 7: 4126–4134.